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(54) Title: COMPOSITIONS, TEST KITS AND METHODS FOR DETECTING HELICOBACTER PYLORI

(54) Titre: COMPOSITIONS, KITS D'EXAMEN ET PROCEDES POUR DETECTER HELICOBACTER PYLORI

(57) Abstract

A composition of specific antigens from Helicobacter pylori for the detection of an infection with this human pathogen and the determination of eradication of the infection after eradication treatment in a biological sample is disclosed. Comparison with other tests proving the infectious status and the eradication of the tested and treated individuals is included to show the useful application of this test.

(57) Abrégé

L'invention concerne une composition d'antigènes spécifiques provenant de Helicobacter pylori, qui est destinée à détecter les infections provoquées par ce pathogène humain et à déterminer si l'infection a été éradiquée au terme d'un traitement d'éradication dans un échantillon biologique. L'invention comprend aussi une comparaison avec d'autres tests prouvant l'état d'infection et l'éradication de l'infection chez les individus soumis au test et au traitement, qui permet de montrer l'utilité de ce test.

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A composition of specific antigens from *Helicobacter pylori* for the detection of an infection with this human pathogen and the determination of eradication of the infection after eradication treatment in a biological sample is disclosed. Comparison with other tests proving the infectious status and the eradication of the tested and treated individuals is included to show the useful application of this test.

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Description

COMPOSITIONS, TEST KITS AND METHODS FOR DETECTING *HELICOBACTER PYLORI*

TECHNICAL FIELD

The invention relates to an antigen composition that can detect the presence of antibodies specific to *Helicobacter pylori*. The invention also relates to a method for the preparation of the antigens and the composition and a method and kit for detecting the presence of the *Helicobacter pylori*-specific antibodies. The method also is able to detect eradication of the organism, providing novel methodology.

BACKGROUND OF THE INVENTION

Helicobacter pylori (formerly *Campylobacter pylori*), hereinafter also referred to as *H. pylori*, was discovered by B.J. Marshall et al. in 1983. It is a gram-negative, spiral shaped, motile bacterium that colonizes the human stomach that more than 50% of the world's adult population in industrial countries and almost 100% in developing countries are infected with. In association with the infection, gastric disorders like chronic gastritis, gastric and duodenal ulcer disease as well as gastric carcinoma occur.

The diagnosis of an infection with *H. pylori* is usually achieved in two ways. Directly (invasive) by endoscopic examination with biopsy, followed by histology and culture of the bacterium and indirectly (non invasive) by testing peripheral blood or serum samples for antibodies against *H. pylori* or performing a ^{13}C urea breath test (UBT).

Serological tests and the ^{13}C UBT are the two non-invasive techniques, used in the management of *H. pylori* infection and eradication. The accuracy of a serological test is dependent on the nature of the antigen(s). Most of the serological tests are ELISA based and use whole cell lysates of *H. pylori* as the antigen, often in combination with a more purified antigen preparation like recombinant *vacA*, *cagA* and/or *iceA* protein. Using a crude lysate preparation of the whole organism can cause problems with the specificity of the test via nonspecific binding of antibodies not specific for *H. pylori* to components of the antigen preparation that might be present in other *H. pylori* related organisms (false positives). On the other hand crude antigen preparation might cause false negative results because unwanted components in the preparation might dilute specific antigens or interfere with the presentation of those required to determine infection. The use of a total protein isolate also prevents serology from detecting loss of the organism and therefore is not suitable for evaluating success of eradication therapy. The UBT gives false negatives when patients are taking PPI's due to inhibition of urease activity by neutral pH.

Currently, the role of serology in managing *H. pylori* infection is as a screening procedure and for diagnosis of infection but not for determination the success of eradication. That is because the tests are not designed to detect reductions in the antibody titer during the post eradication period. In contrast the UBT is highly sensitive and specific but expensive and not available to all general physicians and is not an office procedure. There is an unsatisfied need for an easy non-invasive and sensitive test to both diagnose the infection and to determine eradication of *H. pylori* infection after treatment available as an office procedure to gastroenterologists.

The accuracy of IgG serology, and therefore the usefulness of that approach in monitoring therapy (Hirschl, 1993) and to confirm *H. pylori* eradication has already been pointed out and shown by other authors (Lerang et al., 1998; Cullen et al., 1992, Kosunen et al., 1992). In a recent report an immunodominant outer membrane protein of *H. pylori* has been successfully used to asses the early response to eradication therapy in patients on a serological basis (Nishizono et al., 1998).

The identification of unique *H. pylori* proteins/antigens others than *cagA*, *vacA* and *iceA* that can be used for diagnosis of *H. pylori* infection and for monitoring the success of eradication therapy in patients using a Western blot based method is therefore highly desirable.

United States Patent 5,846,751 is related to a sensitive and specific antigen preparation for the detection of *H. pylori* in biological samples. The preparation uses a range of antigens derived from size exclusion chromatography of detergent-solubilized *H. pylori* cells. United States Patent 5,459,041 discloses an antigenic composition for detecting the presence of antibodies specific for *H. pylori* wherein said antigen is a surface structure resolving into bands migrating at 63,000; 57,000 and 31,000 dalton bands when electrophoresed on sodium dodecyl sulfate polyacrylamide gel. United States Patent 5,859,219 relates to a purified vacuolating toxin from *H. pylori* and methods to use same.

SUMMARY OF THE INVENTION

The subject invention has several distinct aspects. One aspect is a composition of antigens from *H. pylori* present in the lysate of whole bacterial cell preparations that is capable of detecting the presence or absence of specific antibodies against *H. pylori* with high accuracy and reliability. Another aspect is a method for the preparation of such a composition. A further aspect is a method for detecting the presence of antibodies resulting from *Helicobacter pylori* infection in a biological sample which makes use of such a composition. In particular the method relates to monitoring the success of eradication treatment of *Helicobacter pylori*. An additional aspect of the invention is a kit for determining the presence of antibodies formed in response to Helicobacter infection in a biological sample, the kit comprising such a composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B show the average titers of specific antibodies, expressed as percent IOD, with HP1, HP2, HP3 and HP4 from *H. pylori* strain Hp504 (ATCC#43504) present in sera from 9 patients diagnosed with a *H. pylori* infection achieved in two independent experiments. The serum samples from each patient were obtained before, 3 months and 5 months after eradication therapy.

Figure 2 shows the average titers of specific antibodies, expressed as percent IOD, with HP1, HP2, HP3 and HP4 from *H. pylori* strain Hp08 (clinical isolate) present in sera from 9 patients diagnosed with *H. pylori* infection. The serum samples from each patient were obtained before, 3 months and 5 months after eradication therapy.

Figure 3 shows the average titers of specific antibodies, expressed as percent IOD, with HP1, HP2, HP3 and HP4 from *H. pylori* strain Hp02 (clinical isolate) present in sera from 9 patients diagnosed with *H. pylori* infection. The serum samples from each patient were obtained before, 3 months and 5 months after eradication therapy.

Figure 4 summerizes the data shown in the previous figures. It shows the average titers of specific antibodies, expressed as percent IOD, with HP1, HP2, HP3 and HP4 from all three different *H. pylori* strains Hp504, Hp08 and Hp02 present in sera from 9 patients diagnosed with a *H. pylori* infection. The serum samples of each patient were obtained before, 3 months and 5 months after eradication therapy.

DETAILS

The composition according to the invention comprises at least three *Helicobacter pylori* derived proteins or their antigenic regions, wherein the proteins are selected from the group of *Helicobacter pylori* derived proteins which are identified by SDS PAGE to consist of antigens specific to *Helicobacter pylori* of molecular weights 32 kd; 30 kd; 23 kd; and 15 kd and wherein the 15 kd antigen consists of two different *Helicobacter pylori* derived proteins. These antigens from *H. pylori* have not been used in this combination in other available tests. The antigens were characterized by SDS-PAGE as described herein and microsequencing after immunoblotting.

The apparent molecular weights of the antigens (referred to as HP1, HP2, HP3 and HP4; HP4 consists of HP4a and HP4b) calculated according to the molecular weight standards are as follows: HP1 with ~32 kd, HP2 with ~30 kd, HP3 with ~23 kd, HP4a with ~15 kd and HP4b with ~15 kd.

The antigens were identified by determining 20-21 N-terminal amino acids and performing a blast search as follows: HP1 was identified to be the response regulator from *H. pylori* (Tomb et al., 1997), HP2 is the 26 kd antigen from *H. pylori* (O'Toole et al., 1991;Tomb et al., 1997), HP3 is the nonheme

iron-containing ferritin from *H. pylori* (Doig P. et al., 1992; Frazier B.A. et al., 1993), HP4a is the thioredoxin from *H. pylori* (Tomb et al., 1997) and HP4b is a histone-like DNA-binding protein from *H. pylori* (Tomb et al., 1997).

SDS PAGE in connection with the invention refers to SDS-Polyacrylamide Gel Electrophoresis. SDS PAGE preferably is carried out as described under Materials and Method. Before SDS PAGE the antigens preferably are reduced and denatured.

In a preferred embodiment of the invention antigens HP1, HP2, HP3 and HP4 are present in the composition according to the invention.

Preferentially the antigens in the composition according to the invention are present attached to a solid phase. In connection with the invention a solid phase preferably relates to a solid phase suitable for attachment of antigens, such as microtiter plates or membranes such as nitrocellulose and PVDF membranes.

In a preferred embodiment of the invention the composition according to the invention can be achieved by preparing a lysate of whole bacterial cell preparations of *Helicobacter pylori* and subjecting the lysate to gel separation. After separation the antigens may be transferred onto a solid phase, for example by electrotransfer to membranes. In another aspect of the invention the proteins of the composition can be prepared according to recombinant methods. This can be achieved by cloning the complete sequence coding for the antigen(s) or part of it into an appropriate expression vector for an *Escherichia coli* expression system. These systems depend on expression of the protein of interest by induction of a system integrated promoter. After expression of the protein in high amounts it can be isolated and purified by affinity chromatography because it was expressed as a fusion protein or because flag has been attached to it. The possibilities of isolation and purification are entirely depending on the chosen system. In the case that only parts of the sequence of a protein are used for recombinant expression, an antigenicity plot has to be performed to make sure that highly antigenic regions of the protein are not lost thereby loosing the capability of immuno reactivity with the specimen to be tested.

In a further embodiment the invention relates to a method for detecting the presence of antibodies resulting from *Helicobacter pylori* infection in a biological sample comprises the steps of

(a) contacting the sample with a composition according to the invention;
(b) permitting the sample and said composition to form an antigen-antibody complex with respect to any antibody specific for said antigens of the composition contained in the sample;
(c) detecting the presence of any formed antigen-antibody complex denoting the presence of *Helicobacter pylori* infection.

Biological sample in connection with the invention preferably relates to human sera. For detection of the presence of any formed antigen-antibody complex in step (c) it is preferred to use gold label or enzyme conjugated antibody, in particular an anti-Human IgG antibody. The person skilled in the art is familiar what kind of gold label or enzyme conjugated anti-Human IgG antibody can be used in connection with the detection of a said antigen-antibody complex. Anti-Human IgG antibodies which are conjugated to horseradish peroxidase may be mentioned by way of example.

A further embodiment of the invention is a kit for determining the presence of antibodies formed in response to *Helicobacter pylori* infection in a biological sample, the kit comprising a composition according to the invention preferably attached to a solid support. Optionally the kit may comprise additional components such as a positive control (human serum containing antibodies against *H. pylori*), buffer solutions, suitable gold label antibody or an enzyme conjugated anti-Human IgG antibody and a suitable enzyme substrate. In a preferred embodiment of the invention the kit comprises a test strip wherein a composition according to the invention is attached to a nitrocellulose membrane and a suitable gold label antibody is used for detection of the presence of any formed antigen-antibody complex. After contacting the test strip with the biological sample the formation of a coloured line will denote the presence of *Helicobacter pylori* infection. The person skilled in the art is familiar with such type of test strip. This format of test strip is for example widley used is pregnancy hCG tests.

The method for detecting the presence of antibodies resulting from *Helicobacter pylori* infection is particularly suitable for determination of the eradication of *Helicobacter pylori* during and after eradication treatment as it allows to detect reductions in the antibody titer during the post eradication period. This method comprises the steps of:

(a) diagnosis of infection with *H. pylori*;
(b) monitoring antibody titers during eradication treatment;
(c) determination the eradication of the infection after eradication therapy,

wherein at least in steps (b) and (c) the presence or absence of antibodies resulting from *H. pylori* infection is determined by a method according to the invention.

Further subjects of the invention will be evident from the description and the patent claims.

Materials and Methods

Materials: All materials used were of highest purity grade available.

Bacterial strains:

H. pylori strain ATCC#43504 (Hp504)(American Type Culture Collection, Rockville, Maryland) and two clinical isolates, Hp08 and Hp02, were used as the source of *H. pylori* proteins. As a control for the specificity of the serological reactivities *Campylobacter jejuni* strain ATCC#29428 was included into the experiments.

Bacteria were grown on blood agar plates (BBL TSA 5% sheep blood, Becton Dickinson, Cockeysville, MD) for 24 hr or in brain heart infusion (BHI) supplemented with 0.25% yeast extract (Difco Laboratories, Detroit, MI) and 6% horse serum (Gibco BRL, Grand Island, NY) until reaching an OD_{600} of 0.8-1.0 at 37° C in a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). Bacteria grown in broth culture were collected by spinning for 10 min at 5000 x g, washed once with phosphate buffered saline (PBS) pH 7.5 and then suspended in 1 ml ice cold deionized H_2O. Cells grown on up to three blood agar plates were harvested directly into 1 ml ice cold deionized H_2O. Lysis of the cells was obtained by three cycles in a French pressure cell with 20,000 psi at 4° C. The lysates were always kept on ice or at -20° C.

Human sera and antibodies specific for *H. pylori* proteins:

Human sera were provided by Dr. D. Vaira (S. Orsola Hospital, Bologna, Italy). We tested sera from nine with *H. pylori* infected patients (mean age 62.2, 5 female, 4 male; table 1) obtained before, 3 and 5 months after eradication therapy and sera from ten non-infected patients, (mean age 42.6, 5 female, 5 male; table 2) obtained before therapy. The *H. pylori* infection and the status of the gastrointestinal damage of all these individuals had been confirmed and examined by several assays (endoscopy, Clo, Colt, Histology, ELISA ^{13}C UBT; table 1 and 2). The ELISA used for this purpose is described in Literature (Vaira et al., 1988a, 1988b, 1989, 1991, 1994a, 1994b; Oderda et al., 1989a, 1989b, 1991, 1992; Menegatti et al., 1995, 1996, 1998). According to these tests *H. pylori* infection has been eradicated after treatment in all patients.

As controls well characterized and specific polyclonal antibodies against a synthetic peptide of the urease B subunit from *H. pylori* (αUreB#744, Byk Gulden, Konstanz, Germany), the urease A subunit from *H. pylori* (αUreA#30588, Dr. H. Mobley, Univ. of Maryland, Baltimore, MD) and a commercially available antiserum against the Hsp60 from *Synechococcus sp.* strain PCC 7942, (StressGen Biotechnologies Corp., Victoria, BC, Canada) (αHsp) were used. The latter detects specifically HspB of *H. pylori*.

H. pylori eradication therapy:

Substance	Dose
Amoxicillin	1g 2x day
Clarithromycin:	500mg 1x day
Omeprazole:	20mg 1x day
Total duration of treatment:	7 days

Table 1: List of *H. pylori* infected patients

[t] therapy	Patient	Sex	Age		EDGS	Clo A	Clo C	Colt A	Colt C	Histology A	Giemsa A	Histology C	Giemsa C	IgG	^{13}C UBT
before	A3 808	m	45	1st	AG	pos	pos	pos	pos	MIAG atr	1	MAG atr	1	0.94 pos	9.0 pos
				2nd	N	neg	neg	neg	neg	N	0	N	0	n.d.	0.0 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.88 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.70 pos	n.d.
before	A3 828	m	69	1st	N	pos	pos	pos	pos	MIAG	1	MIAG atr	1	1.02 pos	28.3 pos
				2nd	AG	neg	neg	neg	neg	MIAG atr	0	MIAG	0	n.d.	1.7 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.91 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.87 pos	n.d.
before	A3 837	f	49	1st	AG	pos	pos	pos	pos	MIAG	1	MIAG	1	0.79 pos	15.1 pos
				2nd	N	neg	neg	neg	neg	N	0	MIAG atr IM	0	n.d.	1.2 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.65 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.24 neg	n.d.
before	A3 838	f	78	1st	DE	pos	pos	pos	pos	MAG atr	1	N	1	0.96 pos	22.9 pos
				2nd	DE	neg	neg	neg	neg	MIAG atr	0	N	0	n.d.	1.5 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.89 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.56 pos	n.d.
before	A4 012	f	57	1st	DE	pos	pos	pos	pos	MAG atr	1	MAG atr	1	0.83 pos	48.2 pos
				2nd	D	neg	neg	neg	neg	MIAG	0	MIAG atr	0	n.d.	0.0 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.76 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.34 border	n.d.
before	A4 218	f	79	1st	AE	pos	pos	cont	cont	MAG	2	MIAG	2	0.97 pos	71.1 pos
				2nd	N	neg	neg	neg	neg	MICG atr	0	MICG	0	n.d.	1.2 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	lost	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.73 pos	n.d.
before	A4 221	f	50	1st	DE	pos	pos	pos	pos	MIAG	1	MIAG	1	0.95 pos	40.1 pos
				2nd	DE	neg	neg	neg	neg	MICG atr	0	MICG atr	0	n.d.	0.4 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.62 pos	n.d.
before	A4 101	m	60	1st	GU	pos	pos	pos	pos	MIAG	2	MIAG	2	1.01 pos	14.3 pos
				2nd	N	neg	neg	neg	neg	MICG atr IM	0	MICG atr IM	0	n.d.	0
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.77 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.93 pos	n.d.
before	A4 102	m	73	1st	GU	pos	pos	pos	pos	MIAG	3	MIAG	1	0.88 pos	45.1 pos
				2nd	DE	neg	neg	neg	neg	MICG atr	0	MICG	0	n.d.	0.0 neg
3 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.80 pos	n.d.
5 months					n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.85 pos	n.d.

N=normal; AG=antral gastritis; AE=antral erosions; ED=erosive duodenitis; SAG=severe antral gastritis; DU=duodenal ulcer; GU=gastric ulcer

atr=atrophy; IM=intestinal metaplasia; D=dysplasia; MIAG=mild active gastritis; MICG=mild inactive gastritis

m=male; f=female; A=antrum; C=corpus; n.d.=not done; u.e.=under evaluation

Table 2: List of non-infected patients

[t] therapy	Patient	Sex	Age	EDGS	Clo A	Clo C	Colt A	Colt C	Histology A	Giemsa A	Histology C	Giemsa C	IgG	^{13}C UBT
before	A 449	f	56	AG	neg	neg	neg	neg	N	0	N	0	0.14 neg	0.31 neg
before	A 459	m	41	DE	neg	neg	neg	neg	N	0	N	0	0.17 neg	neg
before	A 454	m	33	AG	neg	neg	neg	neg	N	0	N	0	0.17 neg	0.05 neg
before	A 512	f	22	N	neg	neg	neg	neg	N	0	N	0	0.10 neg	0.24 neg
before	A 569	m	52	AG	neg	neg	neg	neg	MIAG atr	0	N	0	0.07 neg	0.94 neg
before	A4 219	m	24	AG	neg	neg	neg	neg	MICG	0	N	0	0.10 neg	neg
before	A4 222	f	23	AG	neg	neg	neg	neg	N	0	N	0	0.10 neg	1.5 neg
before	A4 227	f	58	AG	neg	neg	neg	neg	MICG	0	N	0	0.16 neg	1.4 neg
before	A4 229	f	62	AG	neg	neg	neg	neg	MICG atr	0	MICG	0	0.24 neg	neg
before	A4 233	m	55	AE	neg	neg	neg	neg	MICG	0	N	0	0.27 neg	neg

N=normal; AG=antral gastritis; AE=antral erosions; ED=erosive duodenitis; SAG=severe antral gastritis; DU=duodenal ulcer; GU=gastric ulcer
atr=atrophy; IM=intestinal metaplasia; D=dysplasia; MIAG=mild active gastritis; MICG=mild inactive gastritis
m=male; f=female; A=antrum; C=corpus; n.d.=not done; u.c.=under evaluation

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western-blot Analysis:

The protein content of the bacterial lysates was determined by the method according to Lowry (Lowry et al., 1951) with bovine serum albumin as a standard. The lysates were dried in a speedVac concentrator. Afterwards the pellets were suspended in gel sample buffer (4% SDS, 12% glycerin, 4% β-mercaptoethanol, 0.01% Serva Blue G250 in 50 mM Tris/HCl pH 6.8) and boiled for 10 min. *H. pylori* and *C.jejuni* lysates were separated on 1.0 or 1.5 mm 7.5-16.5% SDS-tricine gradient gels (Schaegger and v. Jagow, 1987). Therefore a 7.5% and a 16.5% acrylamide solution for the separating gel and a 4% acrylamide solution for the stacking gel were prepared according to the scheme presented in table 3. Using a gradient-mixer the gel was poured slowly, but with continuous flow between the glass plates of the gel-sandwich. The separating gel was overlaid with deonized H_2O and allowed to polymerize for 1 hr. Afterwards the water was removed, APS and TEMED was added to the stacking gel solution that was poured on top of the separating gel. A comb with the appropriate number of sample pockets was inserted and removed after polymerisation for 1 hr. The gel-sandwich was placed in an electrophoresis chamber half filled with bottom running buffer (0.2 M Tris-HCl, pH 8.9). The upper compartment was filled with top running buffer (0.1 M Tris base, 0.1 M Tricine, pH 8.25). The protein samples (preparation see above) were loaded into the pockets and electrophoresis was performed with 15 mA constant current over night. Low or broad range prestained molecular mass standards (BioRad, Hercules, CA, USA) were separated in parallel on each gel. The protein pattern after electrophoresis was determined by Silver staining (Heukeshoven, 1985) or Coomassie blue staining. For calculation of molecular weights RFPL scan software (Version 2.01, Scanalytics) was used.

Table 3: Composition of the Tricine gradient gel:

	Stacking gel	Separating gel			
	4%	7.5%		16.5%	
	1.0 mm/1.5 mm	1.0 mm	1.5 mm	1.0 mm	1.5 mm
Acrylamide solution I	1 ml	3.05 ml	5.00 ml		
Acrylamide solution II				6.66 ml	10.0 ml
Gel buffer	3 ml	6.66 ml	10.00 ml	6.66 ml	10.0 ml
Glycerol				1.33 g	2 g
H_2O	8.4 ml	10.3 ml	15.0 ml	5.3 ml	10.0 ml
10% APS in H_2O*	100 µl	40 µl	50 µl	40 µl	50 µl
TEMED	10 µl	3.75 µl	5 µl	3.75 µl	5 µl

Acrylamide solution I: 48% (w/v) acrylamide, 1.5 (w/v) bisacrylamide (BioRad, Hercules, CA, USA) in H_2O

Acrylamide solution II: 46.5% (w/v) acrylamide, 3.0% (w/v) N,N'-methylene-bis-acrylamide (BioRad, Hercules, CA, USA) in H_2O

Gel buffer: 3M Tris-HCl, pH 8.45: 0.3% SDS

APS=ammoniumpersulfate, TEMED=N, N, N', N', tetramethylendiamin, SDS=sodiumdodecylsulphate, * freshly prepared,
** added before the gel is poured

Subsequently the proteins were transferred to nitrocellulose or PVDF membranes (BioRad, Hercules, CA, USA) using 192 mM glycine/25 mM Tris/20% methanol as the transfer buffer. After the electro-transfer onto the membrane, proteins were visualized with Ponceau S solution (0.2% Ponceau S in 3% trichloricacid) to be able to cut the membrane into individual strips for incubation with the different human sera and antibodies. Unspecific binding sites were blocked by incubating for 1 hr in 5% nonfat milk in PBS/0.1% Tween20. Incubation with the human sera (1:500), αUreB#744 (1: 1:85,000 and 1:150,000), αUreA#30588 (1:400,000) and αHsp60 (1:150,000) in 5% nonfat milk in PBS/0.1% Tween20 was performed over night at 4° C. After 4x washing with PBS/0.1% Tween20 the membranes were incubated with a peroxidase labeled anti human/rabbit IgG antibody (1:20,000, American Qualex, San Clemente, CA). After washing 4x with PBS/0.1% Tween20, the reaction was visualized by using the ECL™-system or the ECL™Plus-system (Amersham Life Science Inc., Arlington Heights, IL,) for nitrocellulose membranes or PVDF membranes respectively according to the manufacturers manual. The membranes were exposed to X-ray films (Fuji Medical Systems USA Inc., Stamford, CT,) for time periods between 30 sec and 30 min and developed with a film processor (Konica SRX-101A, Konica Corporation, Japan).

Quantitative Western-blot Analysis

The reactivities of the antibodies in the sera with the antigens of interest were evaluated by imaging the autoradiographs with a Radioanalytic Imaging System (Ambis QuantProbe™ Software, version 4.31) and using RFPLScan® (version 2.01, Scanalytics) for determining the integrated optical density (IOD) of the protein bands of interested using a Gaussian calculation method provided with the program. The IOD of the particular protein band before treatment was set at 100%. The change of the reactivity, reflecting the titer of specific antibodies in the sera, during the post treatment period was calculated compared to the 100% level.

Protein Sequencing:

For microsequencing, proteins were transferred to PVDF membrane (0.2 µm pore size) and visualized by Coomassie blue staining (0.1% Coomassie Brilliant Blue R250 in 45% methanol, 10% acidic acid) and destaining (45% methanol, 10% acidic acid) of the membrane. The protein bands of interest were cut out of the membrane and sequenced with a gas phase sequencer at the UCLA Protein Microsequencing Facility using the Applied Biosystems 475 A system composed of a 470 A sequencer, a 120 A phenyl thiohydantoin analyzer and a 900 A data module. 20-21 sequential amino acids of each

antigen could be determined and this sequence was used afterwards to perform a homology search in the database to identify the protein (table 5).

IDENTIFICATION OF THE *H. PYLORI* ANTIGENS:

After microsequencing 20-21 amino acids of the three antigens of *H. pylori* a blast search in different data bases was performed. All proteins could be identified with 85-100% identity with *H. pylori* proteins. Protein HP4 was a mixture of two components that both could be identified. Table 5 shows the results of that search.

Anti-gen	MW	Sequence	% Iden-tity*	identified as	Accession # / Data Bank
HP1	~32 kd	MIDVLMIEDHIELAEF	87.5 %	Response regulator from *Hp*	024973 AE000537/EMBL
HP2	~30 kd	MLVTKLAPDFKAPAVLG	100 %	26 kd Antigen from *Hp*	2507172 P21762/Swiss-Prot
HP3	~23,5 kd	MLSKDIIKLLWEQVW	85 %	Nonheme iron-containing ferritin from *hp*	477619 A49694/PIR
HP4a	~15 kd	SHYIELTEEWFESTIKKGVA	100 %	Thioredoxin from *Hp*	3024719 P56430/Swiss-Prot
HP4b	~15 kd	MNKAEFIDLVKEAGKYNSKRE	100 %	Histone-like DNA-binding protein from *Hp*	2313970 AE000595/Genbank

* in an 16 - 21 aa overlap

Table 5: *H. pylori* antigens identified by microsequencing

Examples

SDS-PAGE AND IMMUNOBLOT ANALYSIS:

The separation of whole cell lysates of three *H. pylori* strains and one *C. jejuni* strain on tricine gradient gels was performed to show the protein patterns by Coomassie blue or silverstaining of the gels.

The following immunoblot analysis probing the membranes with the ten human sera from non-infected individuals showed that there were only a few proteins of *H. pylori* reacting with these sera. All the proteins reacting with the negative sera were mainly found in the higher molecular weight range and are probably proteins being homologous to proteins from other bacterial species and therefore causing cross reactivities with antibodies generated during infection with *H. pylori*. A similar result was seen with *C. jejuni* proteins supporting that these reactions are supposed to be considered as non specific.

Probing the membranes with the nine human sera from *H. pylori* infected patients obtained before eradication treatment showed again some cross reactivities with *C. jejuni* proteins being either in the high molecular weight range or definitely different from proteins recognized in the tested *H. pylori* strains by these sera.

Using specific sera against both subunits of urease from *H. pylori*, urease A and urease B, and one of the heat shock proteins, HspB, showed that the antigens according the invention are different from these *H. pylori* proteins.

All of the five antigens that are subject of this invention were clearly recognized by eight of the investigated sera from *H. pylori* infected patients in all three tested *H. pylori* strains (Hp504, Hp08, Hp02). One of the sera (HS #012, table 1) did not react with one of the antigens (HP4).

QUANTIFICATION OF THE REACTIVITIES WITH THE *H. PYLORI* ANTIGENS:

The five immuno-reactive antigens described in this invention were visualized by using the ECL™ detection system. The reactivities of the five antigens of interest were evaluated by imaging the autoradiographs with a Radioanalytic Imaging System and using specialized software (RFPLScan© version 2.01) for determining the integrated optical density (IOD) of each single antigen at the different time points (before treatment, 3 months and 5 months after treatment) from the three different *H. pylori* strains. The IOD of each antigen before eradication therapy was set as 100% on each immunoblot that was evaluated. The changes in the reactivities of the sera with these antigens could also be looked at showing the changes in titers of specific serum IgG antibodies against HP1, HP2, HP3, HP4a and HP4b.

The following figures show the serial changes in titers of serum IgG expressed in % of integrated optical density that is left 3 months and 5 months after eradication treatment in comparison to the amount before treatment. The nine sera from with *H. pylori* infected and treated patients were tested on whole cell lysate separations of *H. pylori* strain ATCC#43504 (Hp504), Hp08 and Hp02 (clinical isolates). The results are shown for each tested bacterial strain separately to demonstrate that the accuracy of the test is independent of the source of the antigen.

Figure 1A and B shows the data for the sera being tested on Hp504 antigen preparations in two independent experiments. Differences between the two data sets obtained with antigens from strain Hp504 show that the out come could be depending on the antigen preparation itself and/or the performance of the Western-blot analysis. This points out that those parameters need to be standardized when applying this approach for the development of a commercial available kit. Figure 2 and 3 show the results for the sera being tested on Hp08 and Hp02 antigen preparations respectively.

In all cases there was a significant decrease detected in the reactivities of the nine sera with the five antigens 3 months after therapy that increased further 5 months after treatment. The detected decrease in titers of *H. pylori* specific antibodies shows eradication of the infection what is supported by the results of the other tests that were performed on the patients (table 1).

Figure 4 summarizes the data of the previous experiments and shows the average titers of specific antibodies against HP1, HP2, HP3, HP4a and HP4b from all three different *H. pylori* strains in the patient's sera. As shown here the average titer of anti-HP1 antibodies at 3 months after eradication treatment decreased to 38.5% (= 61.5% reduction) and to 18.03% (= 81.97% reduction) at 5 months after end of treatment respectively. The average titer of anti-HP2 antibodies found at 3 months is down to 49.73% (= 50.27% reduction) and to 31.08% (= 68.92% reduction) at 5 months after therapy respectively. For anti-HP3 antibodies there is a decrease in the average titer to 36.29% (=63.71% reduction) at 3 months and a further decrease to 28.87% (= 79.13% reduction). Finally the average titer of anti-HP4 antibodies at 3 months is down to 31.61% (= 68.39% reduction) and to 14.12% (= 85.88% reduction) at 5 months after therapy.

ACCURACY OF A COMBINATION OF HP1, HP2, HP3 AND HP4 IN A TEST SET:

A combination of the described five antigens from *H. pylori* on a Western-blot test strip applying the correct cut-off setting for each of the antigens provides a sensitive test for the diagnosis of an infection with *H. pylori*, for monitoring the early response to eradication therapy and for determining the eradication of the infection. It is preferred to provide a test strip that contains all of the investigated antigens because the study showed that one or the other of the antigens is recognized differently by the different sera. Providing the combination and not a mixture of HP1, HP2, HP3 and HP4 on a strip also decreases drop-outs if a serum fails to react with one of the antigens. Table 4 shows the cut-off setting for each of the antigens.

Antigen	cut-off at 3 months post therapy	cut-off at 5 months post therapy
HP1	58% titer decrease	78% titer decrease
HP2	44% titer decrease	63% titer decrease
HP3	58% titer decrease	74% titer decrease
HP4	62% titer decrease	84% decrease

Table 4: Cut-off setting for *H. pylori* antigens used in test kit.

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Claims

CLAIMS

1. Composition comprising at least three *Helicobacter pylori* derived proteins, wherein the proteins are selected from the group of *Helicobacter pylori* derived proteins which are characterized by SDS PAGE to consist of antigens specific to *Helicobacter pylori* of molecular weights 32 kd; 30 kd; 23 kd; and 15 kd and wherein the 15 kd antigen consists of two different *Helicobacter pylori* derived proteins.

2. Composition according to claim 1, wherein proteins were isolated and purified.

3. Composition according to claim 1 wherein all said *Helicobacter pylori* derived proteins are present.

4. Composition according to claim 1, which is a combination and not a mixture of said *Helicobacter pylori* derived proteins.

5. Composition according to claim 1 wherein the 23 kd antigen is a nonheme iron-containing ferritin from *Helicobacter pylori*, one of the 15 kd antigens is a thioredoxin from *Helicobacter pylori* and the other 15 kd antigen is a histone-like DNA-binding protein from *Helicobacter pylori*.

6. Composition according to claim 1, wherein the antigen specific to *Helicobacter pylori* of molecular weight 32 kd represents a response regulator from *Helicobacter pylori*.

7. Composition according to claim 1 wherein two proteins forming the 15 kd antigen are present.

8. Composition according to claim 1 wherein the 30 kd, the 23 kd and at least one of the 15 kd antigens is present.

9. Composition according to claim 1, wherein the antigens are present attached to a suitable solid phase.

10. Composition according to claim 9, wherein the solid phase is a microtiter plate.

11. Composition according to claim 1 wherein the antigens are present attached to membranes.

12. Composition according to claim 11 wherein the membranes are nitrocellulose or PVDF membranes.

13. Composition according to claim 9, wherein a combination and not a mixture of all said antigens is provided on a test strip.

14. Method for the preparation of a composition according to claim 12 by preparing a lysate of whole bacterial cell preparations of *Helicobacter pylori*, subjecting the lysate to gel separation and transferring the proteins to the membranes.

15. Method according to claim 14 wherein the *Helicobacter pylori* is *Helicobacter pylori* strain ATCC#43504.

16. Method according to claim 14 wherein the gel separation is carried out in 7.5-16.5% SDS-tricine gradient gels.

17. A method for detecting the presence of antibodies resulting from *Helicobacter pylori* infection in a biological sample, the method comprising

(a) contacting the sample with a composition according to claim 1;
(b) permitting the sample and said composition to form an antigen-antibody complex with respect to any antibody specific for said antigens of the composition contained in the sample;
(c) detecting the presence of any formed antigen-antibody complex denoting the presence of *Helicobacter pylori* infection.

18. A method according to claim 17 wherein in step (c) an enzyme-conjugated anti-Human IgG antibody is used for detection of any formed antigen-antibody complex.

19. A method according to claim 18 wherein the anti-Human IgG antibody is conjugated to horseradish peroxidase.

20. A method according to claim 17 wherein in step (c) gold labled antibody is used for detection of any formed antigen-antibody complex.

21. A method according to claim 17 wherein the biological sample is human serum.

22. A kit for determining the presence of antibodies formed in response to *Helicobacter pylori* infection in a biological sample, the kit comprising a composition according to claim 1.

23. A kit according to claim 22 wherein a combination and not a mixture of all said antigens is provided on a test strip.

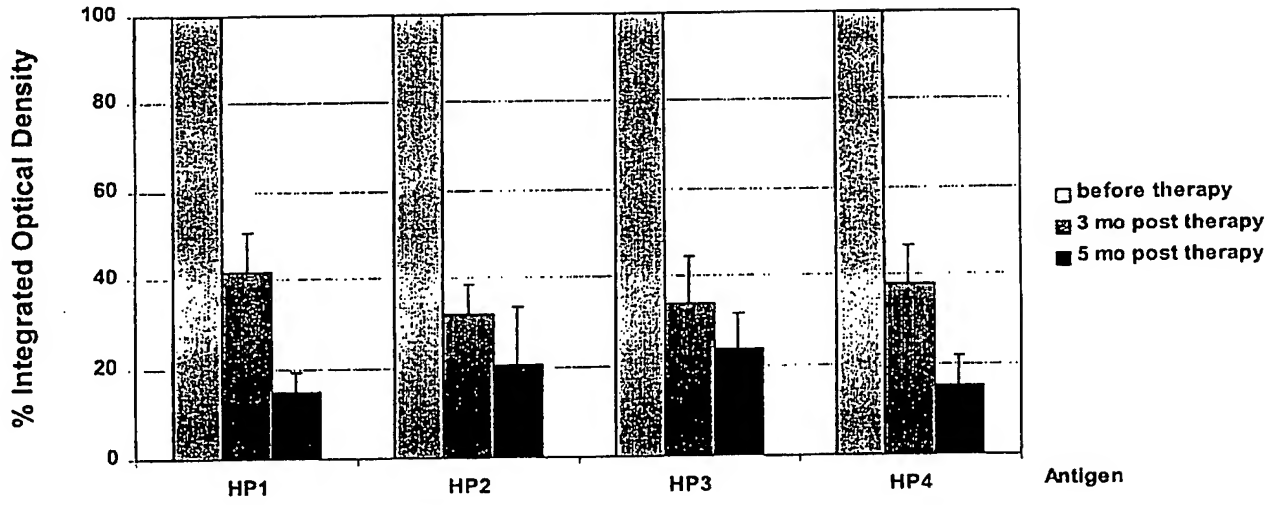
24. A kit according to claim 22 additionally comprising a positive control, an enzyme-conjugated anti-Human IgG antibody optionally a suitable enzyme substrate and buffer solution.

25. A kit according to claim 22 comprising a test strip, wherein a composition according to claim 1 is attached to a nitrocellulose membrane and a gold labled antibody is used for detection.

26. In a method for determination the eradication of *Helicobacter pylori* the improvement consisting in the detection of the presence or absence of antibodies resulting from *Helicobacter* infection by a method according to claim 17, before, during and after eradication treatment.

27. Use of a combination of antigens from *Helicobacter pylori* for detecting the presence or absence of antibodies resulting from *Helicobacter pylori* infection wherein the antigens are selected from the group of *Helicobacter pylori* derived proteins which are characterized by SDS PAGE to consist of antigens specific to *Helicobacter pylori* of molecular weights 32 kd; 30 kd; 23 kd; and 15 kd and wherein the 15 kd antigen consists of two different *Helicobacter pylori* derived proteins.

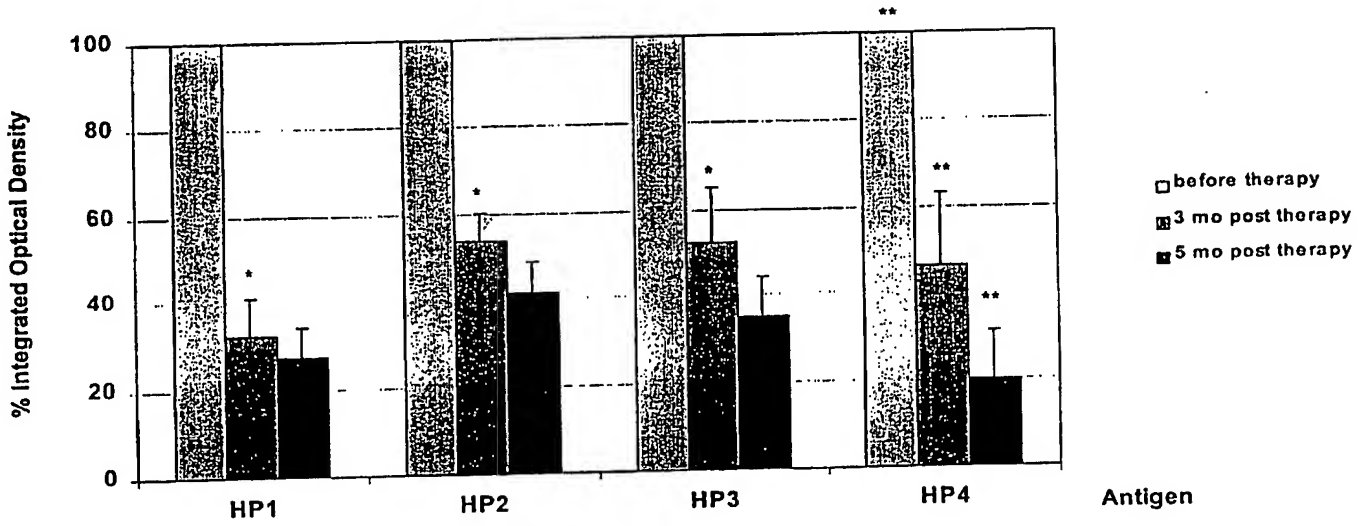
Figure 1A: Reactivities of *H. pylori* positive sera with antigens from Hp504



means +/- SEM, n = 9 for HP2 and HP3; n = 8 for Hp1 and HP4

t/therapy	% IOD HP1	% IOD HP2	% IOD HP3	% IOD HP4
before	100	100	100	100
3 months post	41.61 +/- 9.25	32.08 +/- 6.48	33.88 +/- 10.59	37.86 +/- 8.98
5 months post	15.15 +/- 4.20	20.71 +/- 12.72	23.81 +/- 7.83	15.37 +/- 6.68

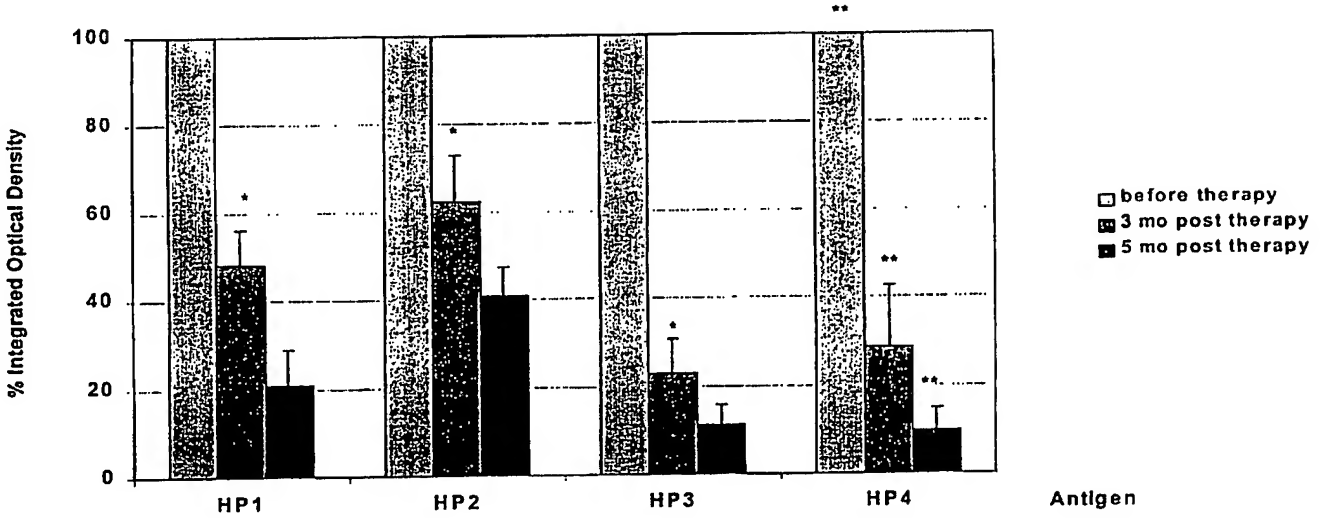
Figure 1B: Reactivities of *H. pylori* positive sera with antigens from Hp504



means +/- SEM, n = 9, *n = 8, **n = 7

t/therapy	% IOD HP1	% IOD HP2	% IOD HP3	% IOD HP4
before	100	100	100	100
3 months post	32.15 +/- 8.85	53.58 +/- 6.81	52.37 +/- 12.72	46.17 +/- 16.87
5 months post	27.2 +/- 7.05	41.28 +/- 7.64	34.77 +/- 9.61	19.77 +/- 11.11

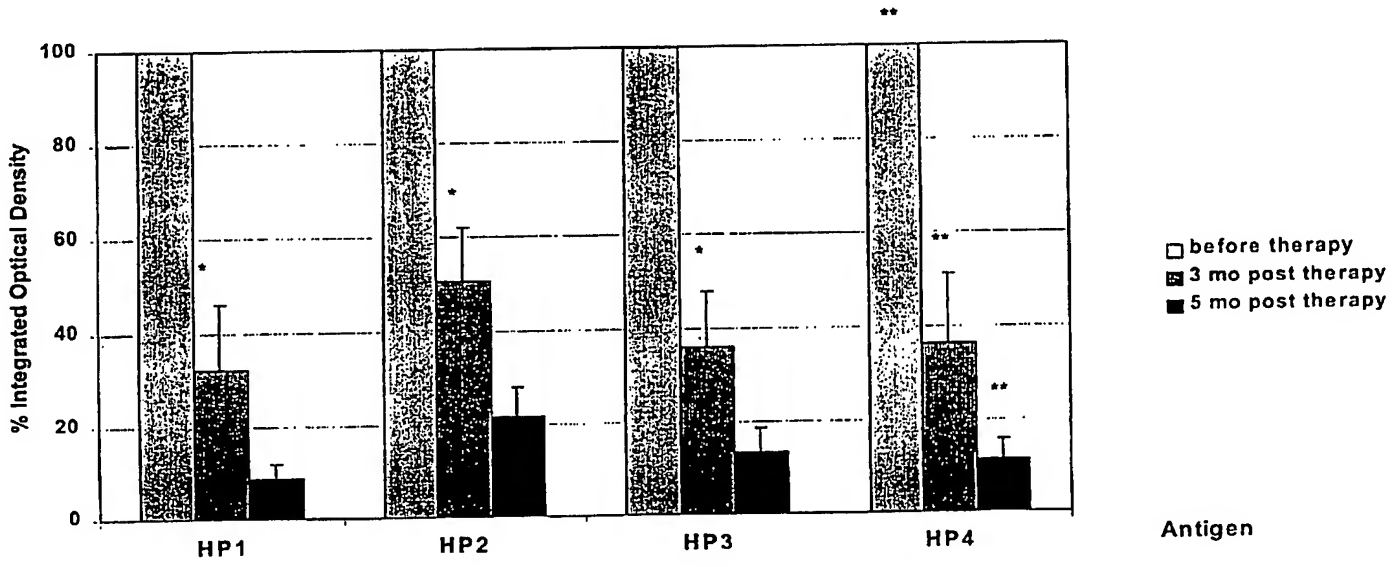
Figure 2: Reactivities of *H. pylori* positive sera with antigens from Hp08



means +/- SEM, n = 9, *n = 8, **n = 7

t/therapy	% IOD HP1	% IOD HP2	% IOD HP3	% IOD HP4
before	100	100	100	100
3 months post	48.11 +/- 8.15	62.53 +/- 10.32	23.05 +/- 7.53	28.60 +/- 13.85
5 months post	21.06 +/- 8.01	40.77 +/- 4.45	11.36 +/- 4.45	9.96 +/- 4.99

Figure 3: Reactivities of *H. pylori* positive sera with antigens from Hp02



means +/- SEM, n = 9, *n = 8, **n = 7

t/therapy	% IOD HP1	% IOD HP2	% IOD HP3	% IOD HP4
before	100	100	100	100
3 months post	32.30 +/- 13.97	50.71 +/- 11.48	35.86 +/- 11.88	36.29 +/- 14.99
5 months post	8.70 +/- 3.2	21.56 +/- 6.44	13.51 +/- 5.02	11.39 +/- 4.27

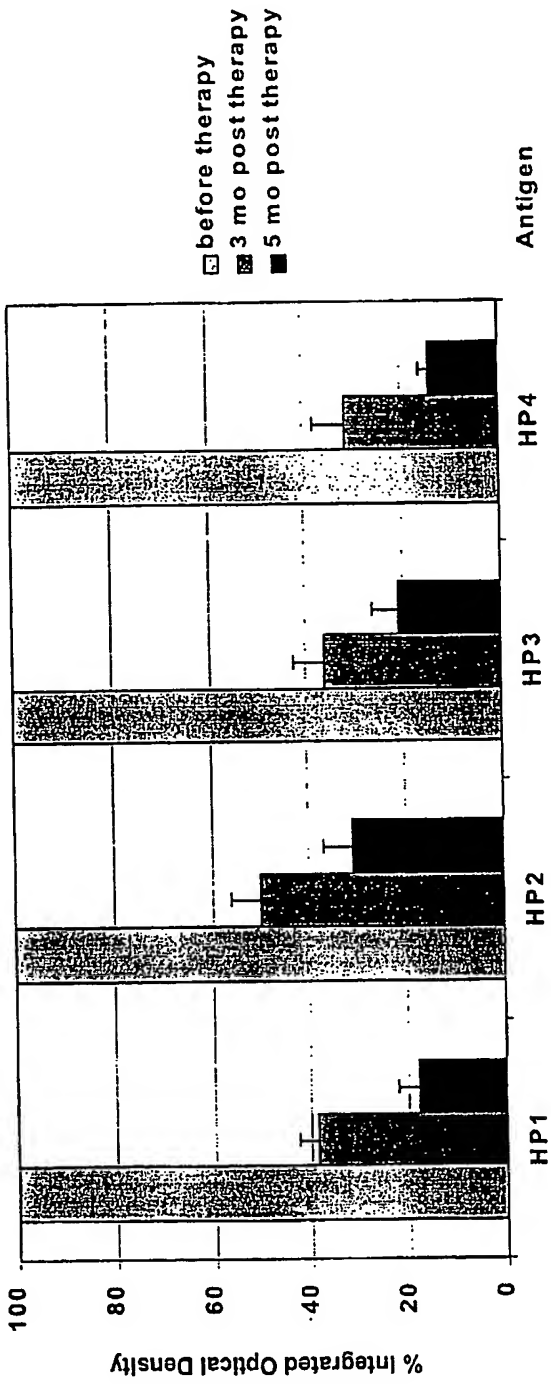


Figure 4: Reactivities of *H. pylori* positive sera with antigens from Hp504, Hp08 and Hp02

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(54) Title: COMPOSITIONS, TEST KITS AND METHODS FOR DETECTING HELICOBACTER PYLORI

(54) Titre: COMPOSITIONS, KITS D'EXAMEN ET PROCEDES POUR DETECTER HELICOBACTER PYLORI

(57) Abstract

A composition of specific antigens from Helicobacter pylori for the detection of an infection with this human pathogen and the determination of eradication of the infection after eradication treatment in a biological sample is disclosed. Comparison with other tests proving the infectious status and the eradication of the tested and treated individuals is included to show the useful application of this test.

(57) Abrégé

L'invention concerne une composition d'antigènes spécifiques provenant de Helicobacter pylori, qui est destinée à détecter les infections provoquées par ce pathogène humain et à déterminer si l'infection a été éradiquée au terme d'un traitement d'éradication dans un échantillon biologique. L'invention comprend aussi une comparaison avec d'autres tests prouvant l'état d'infection et l'éradication de l'infection chez les individus soumis au test et au traitement, qui permet de montrer l'utilité de ce test.

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(54) Title: COMPOSITIONS, TEST KITS AND METHODS FOR DETECTING *HELICOBACTER PYLORI*

(57) Abstract: A composition of specific antigens from *Helicobacter pylori* for the detection of an infection with this human pathogen and the determination of eradication of the infection after eradication treatment in a biological sample is disclosed. Comparison with other tests proving the infectious status and the eradication of the tested and treated individuals is included to show the useful application of this test.

INTERNATIONAL SEARCH REPORT

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IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANDERSEN L P ET AL: "Isolation and Preliminary Evaluation of a Low-Molecular-Mass Antigen Preparation for Improved Detection of Helicobacter pylori Immunoglobulin G Antibodies." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 2, no. 2, 1995, pages 156-159, XP000917860 ISSN: 1071-412X abstract page 156, column 1, paragraph 2 page 157, column 1, paragraph 1 -column 2, paragraph 1 figure 1 page 157, column 2, paragraph 5 -page 158, column 2, paragraph 2 -/--	5-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 August 2000	12.09.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montrone, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02419

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 26740 A (ENTERON LIMITED PARTNERSHIP) 6 September 1996 (1996-09-06) abstract page 6, line 25 -page 7, line 1 page 11, line 4 - line 16 page 19, line 21 - line 37 tables 3,4	5-25
Y	US 5 846 751 A (PAWLAK JAN WACLAW ET AL) 8 December 1998 (1998-12-08) abstract column 3, line 29 - line 34 column 5, line 17 - line 30 table 1	5-25
Y	AUCHER P ET AL: "Use of immunoblot assay to define serum antibody patterns associated with Helicobacter pylori infection and with H. pylori-related ulcers." JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 4, April 1998 (1998-04), pages 931-936, XP000917831 ISSN: 0095-1137 abstract page 934, column 2, paragraph 2	5-25
A	NISHIZONO AKIRA ET AL: "Serological assessment of the early response to eradication therapy using an immunodominant outer membrane protein of Helicobacter pylori." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 5, no. 6, November 1998 (1998-11), pages 856-861, XP000917721 ISSN: 1071-412X abstract page 857, column 2, paragraph 1 - paragraph 3 page 860, column 1, paragraph 2 -column 2, paragraph 3	26

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02419

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	VOLAND PETRA ET AL: "Identification of antigens of Helicobacter pylori that can be used as markers for diagnosis and monitoring eradication in patients based on a serological method." GASTROENTEROLOGY, vol. 116, no. 4 PART 2, April 1999 (1999-04), pages A346-A347, XP002145286 Digestive Disease Week and the 100th Annual Meeting of the American Gastroenterological Association;Orlando, Florida, USA; May 16-19, 1999 ISSN: 0016-5085 abstract	5-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 00/02419

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-4, 27
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-4, 27

Present claims 1 to 4 and 27 relate to a composition defined by reference to the following parameter:

P1: A composition of five Helicobacter pylori proteins which are only defined by their approximate molecular weight as determined by SDS PAGE.

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the subject-matter of claim 5 combined with the peptide sequences as disclosed on page 12, table 5 of the description.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/02419

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9626740 A	06-09-1996	AU 5523196 A	18-09-1996
US 5846751 A	08-12-1998	US 5814455 A	29-09-1998
		DE 4139840 A	11-06-1992
		FR 2669929 A	05-06-1992
		IT 1252164 B	05-06-1995
		JP 5264553 A	12-10-1993